DRUG DISCOVERY

Anti-inflammatory activity of fractions and isolated compounds from *Tabebuia hypoleuca* stems in mice

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General Note

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discovery

ABSTRACT

In the literature, there are few studies on the species *Tabebuia hypoleuca*. So far, only the anti-inflammatory activity of stems methanol extract is reported in two models of acute inflammation. In the present study we evaluated the anti-inflammatory activity

in the acute phase of the fractions and isolate the active compounds of *T. hypoleuca*. The acute anti-inflammatory activity was evaluated for fractions separate from the total methanol extract and by the major compound isolated using the *in vivo* model of carrageenan-induced paw edema in mice, and the edema percent inhibition was calculated at different times. Fractions and isolating compound were orally administered at doses of 50 mg/kg. TT4 (ethyl acetate-methanol) fraction showed the higher anti-inflammatory activity at doses of 50 mg/kg for all the times tested (1.5, 3, 4.5, 6 and 24 hours), depending on the model of carrageenan-induced paw edema, with 31.40, 48.00, 56.88, 35.22 and 40.54 % inhibition respectively. The other fractions studied showed no significant anti-inflammatory activity. The major compound isolated was a mixing of hopenone b, taraxerone and lupenone. These results demonstrated the anti-inflammatory activity of the fraction TT4 (ethyl acetate-methanol) and the additive activity from hopenone b, taraxerone and lupenone as major composition obtained from the methanol extract from *T. hypoleuca* stems.

Keywords: *Tabebuia hypoleuca*, Bignoniaceae, anti-inflammatory, carrageenan-induced paw edema, hopenone b, taraxerone, lupenone.

1. INTRODUCTION

Inflammation is a complex and delicate mechanism composed of cellular immunity and biochemical mediators with interrelated biological effects that occur as a response to injury, infection, and stress [1, 2]. However, the excessive production of inflammatory mediators during chronic inflammation contributes to the pathogenesis and development of some diseases such as cardiovascular and bowel diseases, cancer, diabetes, arthritis, and neurodegenerative disorders that affect a significant part of the human population [3, 4]. Steroids and non-steroidal anti-inflammatory drugs are the most clinically important drugs to treat inflammatory diseases, which are associated with a high incidence of adverse effects [5]. This justifies the research addressed to study and identify new safer and more effective active substances to prevent and treat inflammatory disorders and related conditions. Throughout history, nature, especially plants, has provided a source of medicines for the treatment of a wide spectrum of diseases [6].

Tabebuia spp. (*Bignoniaceae*) includes approximately 100 species, known as strictly woody, found in tropical rain forest areas throughout Central and South America [7]. Species of the genus *Tabebuia* have been traditionally used for the treatment of several diseases, including syphilis, fever, malaria, cutaneous infections, stomach disorders [8]. Several properties have been reported for species of the genus *Tabebuia*, for instance: anti-fungal, antiparasitic, anti-bacterial, antineoplastic, analgesic, antimalarial, anti-oxidant, anti-cancer and anti-inflammatory [9, 10, 11, 12, 13, 14, 15, 16, and 17].

Tabebuia hypoleuca (C. Wright) Urb. commonly known as "Roble macho" is a species endemic of Cuba and native to Sierra Maestra and Guantanamo. In our previous report, the anti-inflammatory activity of the methanolic extract from *T. hypoleuca* stems has been demonstrated using the carrageenin-induced paw edema models and the croton oil induced auricular edema in mice [18].

The aim of this study was to determine the *in vivo* anti-inflammatory activity of the fractions and isolated compounds obtained from the methanolic extract of *T. hypoleuca* stems, using the carrageenin-induced paw edema model in mice.

2. MATERIAL AND METHODS

Plant material

T. hypoleuca was collected at the National Botanical Garden (JBN), Havana province. The identification of the plant was confirmed by Dr. R. Eldis Becquer and the sample was deposited in the herbarium of the experimental station with the number HFC-88204.

Extraction, fractionation and isolation

Stems were separated and dried at room temperature for one week and after at 37ºC. It was milled to 40 mesh. The total extract was obtained from 330 g of *T. hypoleuca* stems by solid-liquid extraction in Soxhlet with methanol (1 L) (Merck) (70° C, at a rate of siphoned every 20 min for 12 hours). The methanol extract was filtered and concentrated by rotary evaporation. Subsequently, 21.92 g of the extract was chromatographed over silica gel (70 – 230 mesh, 100 g, Merck), eluted first with petroleum ether (500 mL). The eluent polarity was increased by gradients with different solvents: petroleum ether: ethyl acetate, 1:1 (1 L); ethyl acetate (1 L); ethyl acetate: methanol (1 L); methanol (1 L) and methanol: water, 1:1 (1 L). Finally, six fractions were collected (TT1, TT2, TT3, TT4, TT5 and TT6, respectively), and were monitored by TLC in the system ethyl acetate: methanol: water, 7:2:1, UV light and I_2 as revelators were used. Fractions were concentrated by rotary evaporation for bioactivity evaluation.

Compounds present in major fraction, TT2 (petroleum ether: ethyl acetate, 1:1, 2.5 g) was isolated using semi- flash chromatography on silica gel 60 (0.063- 0.200 mm, 1.5 x 45 cm). The column was eluted with hexane, hexane- ethyl acetate

increasing in polarity, ethyl acetate and ethyl acetate- methanol polarity increasing and methanol, finally.   I were collected 32 fractions from 100 ml each.

Compounds present in more active fraction, TT4 was isolated by filter column chromatography with silica gel and eluted with hexane, dichloromethane and dichloromethane – methanol, methanol and methanol – water, increasing the polarity. In this case were collected 42 fractions from 100 ml each.

The fractions was followed by TLC with dichloromethane- methanol 5% or ethyl acetate- methanol – water (7:2:1) and visualized with anisaldehyde (50 mL acetic acid, 0.5 mL sulfuric acid, and 0.5 mL anisaldehyde). More important fractions obtained from TT2 and TT4 were analyzed by GC/MS.

GC–MS analysis
The GC/MS analysis was carried out using an HP-6890/5975 system equipped with an HP-5 (30m× 0.25mm×0.25 μm). Temperature program: 110°C (5°C/min) – 280°C (60 min), injector 250°C, detector 280°C. Helium was used as a carrier gas (0.7 bar, 1mL/min). The MS were taken at 70 eV. Scanning speed was 0.84 scans s−1, from 40 to 550. Sample volume was 1 μL. Split: 1: 40. The mass spectra were compared with previously reported data with software MSD ChemStation D.02.00.275, 1989 – 2005.

Animals
Male BALB/c mice weighing 22 ± 2 g were supplied by the National Center for Laboratory Animal Production (CENPALAB, Santiago de Las Vegas, Havana, Cuba). The mice employed were randomly distributed for tests. Prior to the experiment, animals were adapted to laboratory conditions for seven days (controlled temperature 23 ± 2°C, relative humidity 40-60%, 12 h light/dark cycles with food and water *ad libitum*).

Experimental design
All studies were in compliance with the Good Laboratory Practice (GLP) standards (US, 2001). The experiments were conducted in accordance with the ethical guidelines for investigations in laboratory animals and were approved by the Ethical Committee for Animal Experimentation of the National Center for Animal and Plant Health (CENSA), Havana, Cuba (Cuba, 2004) (approved protocol 01/2017).

Carrageenan-induced paw oedema in mice
The carrageenan-induced paw edema assay was carried out according to the method of Winter et al. (1962) and later modified for mice by Sugishita et al. (1981) [40]. Groups of eight mice were injected with 40 μL of a 3 % carrageenan solution in the sub-plantar region of the right hind paw.

The test groups were orally treated with 50 mg/kg b.w of the fractions, one hour prior to carrageenan injection. At the same time, the control group received the vehicle (saline 0.9 % + Tween 80), while the standard reference group was treated with an indomethacin solution (10 mg/kg, 99% purity) orally. In all cases a volume of 0.02 mL/g was administered. The paw volume was measured by the digital caliper at 1.5, 3, 4.5, 6 and 24 hours after carrageenan administration. The percent reduction in paw volume was calculated according to the following formula [41]:

$$\text{Percentage inhibition} = \frac{(C_t - C_0)\text{ control} - (C_t - C_0)\text{ treated}}{(C_t - C_0)\text{ control}} \times 100$$

C_t = Mean paw edema in each time
C_0 = Mean paw edema at time zero

Is defined as selection criterion a percentage inhibition to edema greater than or equal to 20% [42].

Statistical analysis
Statistical analysis was performed using the statistical software package SPSS (version 21.0). The data were expressed as mean ± SEM. One-way ANOVA followed by the Duncan´s Test was applied to determine the significant differences between the control and treated groups. Values of $p<0.05$ were considered statistically significant.

3. RESULTS

The anti-inflammatory activity of the fractions TT1 to TT6 obtained from the methanolic extract of the stems was evaluated by the carrageenin induced paw edema model. The results showed that the TT4 fraction in dose of 50 mg/kg significantly reduced carrageenan-induced edema at all times. The other fractions studied showed no significant differences in the dose evaluated relative to the negative control group. In the group treated with indomethacin (10 mg/kg) there was a decrease of edema in all times evaluated with respect to the negative control group (Fig. 1). The TT4 fraction showed a maximum reduction percent on carrageenan-induced paw edema at 3 to 4.5 hours, with an inhibition of 48.00 and 56,88 % respectively (Table 1).

Table 1 Percent reduction of the fractions obtained from the methanol extract of the stems of *T. hypoleuca* (TT), on carrageenan induced paw edema in mice.

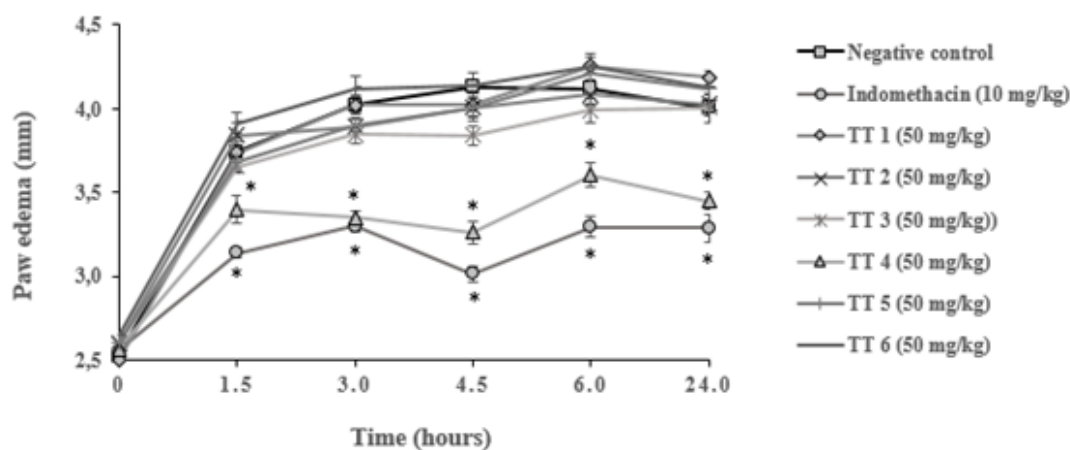| Treatment	Dose (mg/kg)	% Inhibition (hour)				
		1.5	3.0	4.5	6.0	24.0
Control	-	(0)	(0)	(0)	(0)	(0)
TT 1	50	N.E	N.E	4.37	N.E	N.E
TT 2	50	N.E	13.33	11.88	6.29	3.38
TT 3	50	13.22	17.33	23.13	13.21	5.41
TT 4	50	31.40	48.00	56.88	35.22	40.54
TT 5	50	9.92	13.33	11.88	N.E	N.E
TT 6	50	N.E	2.00	6.88	N.E	N.E
Indomethacin	10	52.07	50.67	71.25	53.46	50.68

N.E= no effect.

Figure 1 Effect of oral administration of fractions (50 mg/kg) obtained from the methanolic extract of *T. hypoleuca* stems (TT) and indomethacin (10 mg/kg) on the paw oedema induced by intraplantar injection of 3% carrageenan in mice. The values are expressed as mean ± S.E.M, n = 8, *$p < 0.05$ *vs* negative control (vehicle) group by one-way ANOVA followed by the Duncan´s Test.

From TT2 was isolated 280 mg from compound titled TT2-11 as amorphous white solid, correspondence by a spot with Rf= 0.77, pink color with anysaldehyde. GC/MS from TT2-11 shown 66.9 % purity and 0.61% of yield. Though TT2-11 was a mixture of compounds with a high concentration of   triterpene, hopenone b (isopropenil 5a,5b,8,8,11ª,13b-hexamethylcasahidro-9H-ciclopenta[a]crisen-9-one; A-neogommacer- 22(29)-en-3one) with a molecular formula $C_{30}H_{48}O$. This major compound presented in the chromatogram a retention time of 47.64 minute.

Other peaks were observed at retention times, Rt= 44.98 min (9.04%) y Rt= 42.98 min (6.46%), correspondence at lupenone (lu-20(29)-en- 3 one) and D- friedoolean-14-en-3-one (taraxerone, tarexerol), respectively (Figure 2).
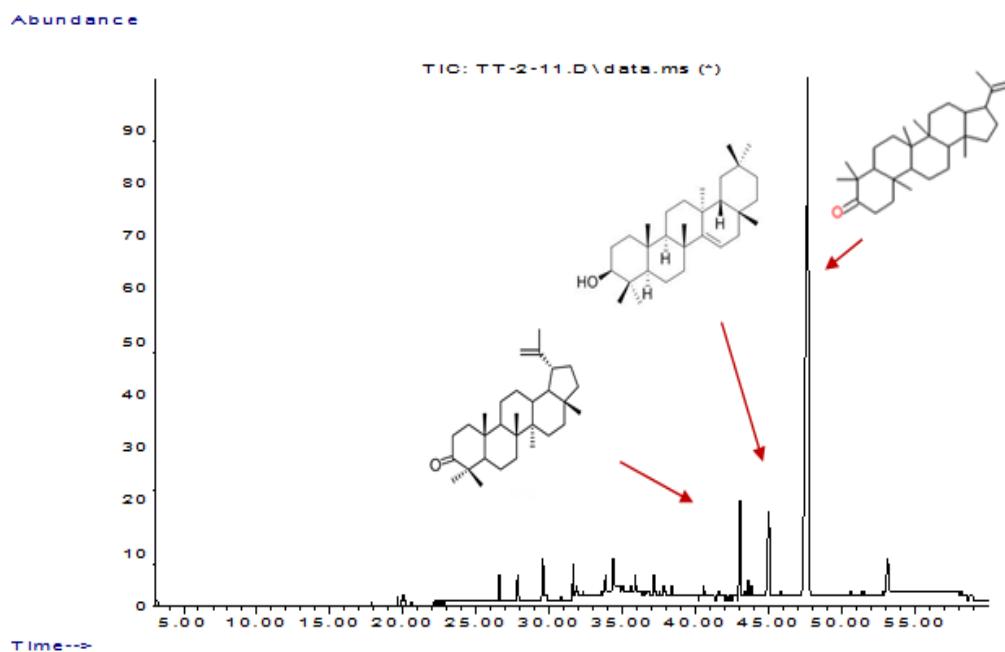
Figure 2 Fingerprint of TT2-11 analyzed by GC–MS.

Figure 3 showed chromatographic profile from TT4 fraction after it separation by column chromatography. GC- MS analysis shown as more important compounds in the mixture at 1,2-benzenedicarboxilic acid, ester; hopenone b; hexanedioic acid, bis(2-ethylhexyl) ester ; nonanedioic acid, dimethyl ester ; (10.27%; 8.32%; 7.91%; 7.73%, respectively, Table 2)
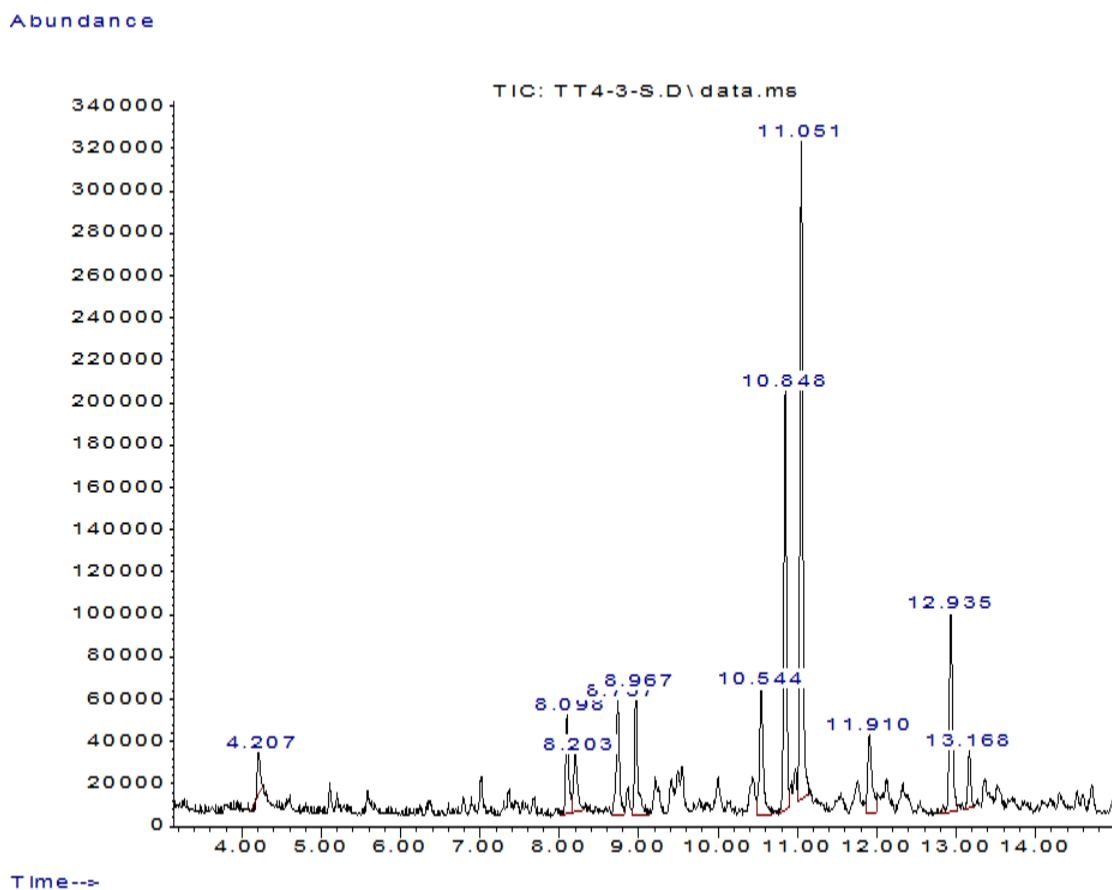

Figure 3 Chromatographic profile from TT4

Table 2 Compounds identifies in TT4 by GC/MS, comparing mass spectra of each peak with NIST- 211 date base

Rt (min)	Identificación	% rel.
10.85	2-butenedioic acid (Z)-, dibutyl ester	4.68
11.05	nonanedioic acid, dimethyl ester	7.73
17.71	1,2-benzenedicarboxilic acid, ester	4.10
19.66	1,2-benzenedicarboxilic acid, ester	5.40
20.84	M = 236	4.17
21.55	1,2-benzenedicarboxilic acid, ester	10.27
27.12	hexanedioic acid, bis(2-ethylhexyl) ester	7.91
47.13	A'-neogammacer-22(29)-en-3-one	8.32

The anti-inflammatory activity of the TT2 (11) obtained from the methanolic extract of the stems was evaluated by the carrageenin induced paw oedema model. The results showed that the (Fig. 4) maximum reduction percent on carrageenan-induced paw oedema at 3 to 4.5 hours, with an inhibition of 48.00 and 56.88 % respectively (Table 3).
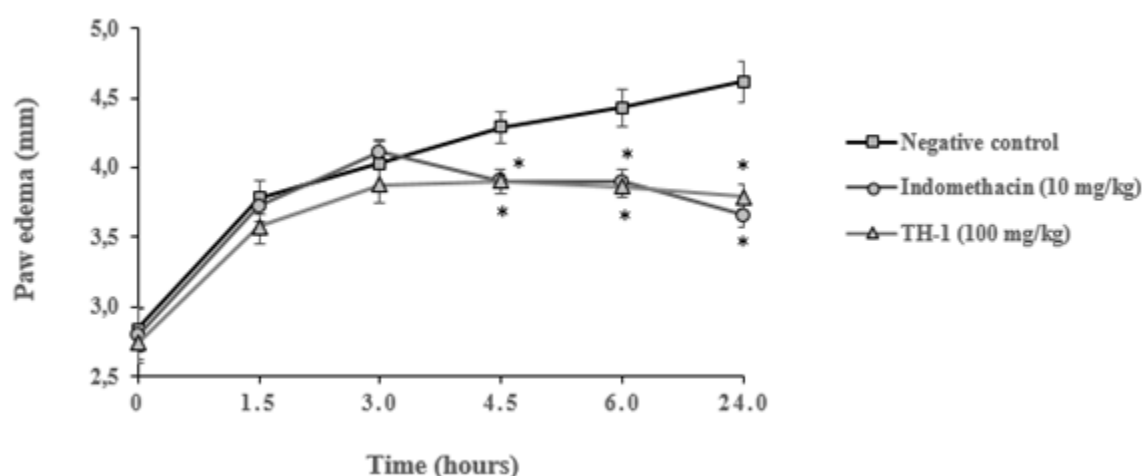

Figure 4 Effect of oral administration of TH-1 mixture (100 mg/kg) and indomethacin (10 mg/kg) on the paw oedema induced by intraplantar injection of 3% carrageenan in mice. The values are expressed as mean ± S.E.M, n = 8, *p < 0.05 vs negative control (vehicle) group by one-way ANOVA followed by the Duncan´s Test.

Table 3 Percent reduction of TH-1 mixture on carrageenin induced paw oedema in mice.

Treatment	Dose (mg/kg)	% Inhibition (hours)				
		1.5	3.0	4.5	6.0	24.0
Control	-	(0)	(0)	(0)	(0)	(0)
TT2 (11)	100	11.64	4.32	21.98	29.46	40.86
Indomethacin	10	1.46	N.E	23.34	30.40	51.41

N.E= no effect.

4. DISCUSSION

Throughout most of history, nature, especially plants, has provided a source of medicines for the treatment of a wide spectrum of diseases. The present study was designed to investigate the anti-inflammatory activity of fractions and compounds of *T. hypoleuca* using the carrageenan-induced paw oedema. This model is widely used to determine the anti-inflammatory activity. It has been fully characterized in the past and is widely used as a working model of inflammation in the search for new anti-inflammatory drug [19, 20].

The inflammatory process is characterized invariably by a production of prostaglandins, leukotrienes, histamine, bradykinin, platelet-activating factor and by the release of chemicals from tissues and migrating cells. The acute inflammatory response is characterized by an increase in the vascular permeability and cellular infiltration leading to edema formation, as a result of an extravasation of fluids and proteins and accumulation of leukocytes in the inflammatory site. The carrageenan edema model in mice has two distinct phases: the early phase starts immediately after carrageenan injection and it lasts for about 6 h while the late phase starts after 6 h and it ends about 72 h after carrageenan injection. Serotonin, phospholipase A2 (PLA_2), histamine, kinins, arachidonate metabolites (prostaglandins, leukotrienes), and nitric oxide (NO) have been strongly linked to the inflammatory process in the early phase [21].

These results suggest that the anti-inflammatory response to 50 and 100 mg/kg of TT4 fraction and TT2- 11 respectively could be likely linked to the inhibition of histamine, bradykinin, PLA2, serotonin release agents, as well as to a decrease in the production of prostaglandins.

Indomethacin response as nonsteroidal anti-inflammatory drug (NSAID) was correspondence with the action mechanism reported by this drug. The mechanistic role of indomethacin in inhibition of pain has been verified by being nonselective inhibitor to cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isozymes. The enzymatic activity of COX involves bis-oxygenation of arachidonic acid to (prostaglandin G2) PGG2, which then reduced to PGH2 in a peroxidase reaction by the same protein [22].

The higher inflammatory percentages in our study were shown in some groups including the negative group with saline. In this case the maxima inflammation was at 4.5 h in correspondence with the carrageenan administration [19, 23]. Preliminary phytochemical study of the methanolic extract from *T. hypoleuca* stems suggested the presence of tannins, phenols and alkaloids [18]. However, in this study, spectroscopic and mass spectrometry data suggest triterpene structures. Major component in the stem titled TT2-11 was a mix of hopenone b (66.9 %), lupenone (9.04 %) and taraxerol (6.46%). Also, the anti-inflammatory activity found in our experiment corresponded with 66.9 mg/Kg (hopenone b), 9.04 mg/Kg of lupenone and 6.46 mg/Kg of taraxerone.

Hopenone b, name moretenone is found in fats and oils. Moretenone is a constituent of *Sapium sebiferum*. It was isolated and associated with *in vitro* antitumor activity from *Pericampylus glaucus* (Lam.) Merr by Wei and Cheng, 2009 [24]. This compound was isolated and evaluated it activity from plants as *Maprounea guianensis* aerial parts [25], from *Euphorbia mellifera* MeOH extract [26], and from the MeOH extract of *Sebastiania schottiana* roots [27] and from *Cnidoscolus chayamansa* [28]. Moretenone was described as an analgesic and anti-inflammatory compound; the analgesic effect was higher than that of aspirin and paracetamol when determined by acetic acid induced abdominal writhes model (in male mice). The analgesic or anti-nociception activity (determined on acetic acid abdominal constrictions in mice) for moretenone was 72% inhibition; it was more active than aspirin and paracetamol (35 and 38% inhibition, respectively); on formalin assay, moretenone was more active (16 and 36% of inhibition at 1st and 2nd phases) than IND (6.6 and 33%, respectively) [27, 29, 30].

Pérez et al., 2017 [28] demonstrated the anti-inflammatory activity of triterpenes compounds on acute inflammation, between these moretenone and Pérez et al., 2018 [31] demonstrated the anti-inflammatory activity on chronic inflammation models. Taraxerol is an anti-inflammatory triterpene that has been isolated from a variety of plants, and have shown the property of decreasing the production of NO, PGE2, NF-kB, TNF-α, IL-6 and IL-1β in vitro [32]. Besides, the anti-inflammatory activity of taraxerol on TPA-induced ear edema model in mice has been reported [33]. The inhibitory effect of taraxerone on nitric oxide generation was significantly more effective than that of caffeic acid and/or gallic acid. The modulation of NO production by inhibiting iNOS expression is potentially therapeutic in relation to inflammation, for also taraxerone has anti-inflammatory ability via antioxidant power. Thus, taraxerone could be applied to the treatment of NO mediated diseases as well as an antioxidant [34]. Lupenone, is a typical polar lupane type triterpenoid and it molecule has gained the attention of medical professionals and researchers for its wide ranging pharmacological activities. Lupenone is a therapeutic and chemopreventive agent for the treatment of inflammation, virus infection, diabetes, cancer, and Chagas disease. Lupenone could inhibit the TPA induced inflammation in mice, could reduce the nitric oxide (NO) and reactive oxygen species (ROS) production, as well as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein levels in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells, indicating that lupenone may be used to therapy and prevent various inflammatory diseases and oxidative stress-related diseases [35, 36, 37].

Xu et al., 2019 [38] evaluated the lupenone activity on acute, subacute and diabetic pancreatic inflammation. These authors found that lupenone significantly decreased acute and subacute inflammation in mice as well as the IL-1β and IFN-γ levels in the pancreas of diabetic rats. These finding provide strong support for studying the molecular mechanism of lupenone in the treatment of diabetes from the perspective of anti-inflammation. The compounds identified in TT4 fraction had a proportion of carboxylic acid ester. The carboxylic acid group (–COOH) is present in classical non-steroidal anti-inflammatory drugs (NSAIDs), for example paracetamol, naproxen, ibuprofen and flurbiprofen had been associated to gastrointestinal toxicity. Because of this, the scientific literature describes the design of a high number of NSAID prodrugs (mainly ester and amide derivatives). These derivatives are

effective and safe anti-inflammatory agents [39]. The anti-inflammatory activity shown for the stem of *Tabebuia hypoleuca* possible is a synergic effect of the different compounds identified in these fractions.

Future studies must demonstrate the activity of pure compounds and their mechanisms of action identify other compounds in TT4 fraction and verified the synergic effect of compounds, and the in vivo efficiency of standardized extracts or pure compounds.

The present study demonstrated anti-inflammatory activity of fraction TT 4 and TT2- 11 mixture from *Tabebuia hypoleuca* stems on carrageenan-induced paw edema in mice (acute phase). There are experiments in progress to identify other bioactive components and to evaluate the anti-inflammatory activity in the chronic phase. This species and their constituents could represent in the future a new therapeutic option for the treatment of inflammatory diseases.

Conflict of Interest: The authors declare that there are no conflicts of interests.

Peer-review: External peer-review was done through double-blind method.

Data and materials availability: All data associated with this study are present in the paper.

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